RECOMBINANT ANTI-GPIIB/IIIA ANTIBODIES

DESCRIPTION

The invention relates to novel nucleic acid sequences which encode human autoantibodies against blood platelet membrane proteins and which encode antiidiotypic antibodies, to novel amino acid sequences of human antibodies, and to their use for the diagnosis and therapy of diseases.

Autoimmune thrombocytopenic purpura (AITP) is an immune disease which is defined by a low blood platelet count associated with normal or elevated megakaryocytopoiesis. The destruction of platelets in the reticuloendothelial system (spleen, liver and bone marrow) is increased due to the presence of anti-platelet autoantibodies. These autoantibodies, which can be detected in about 75% of AITP patients, are predominantly directed against the platelet membrane glycoproteins (GP) IIb/IIIa and Ib/IX. Several different autoantibody specificities may be found in one and the same patient (cf., e.g., Berchtold and Wenger, Blood 81 (1993), 1246-1250; Kiefel et al., Br. J. Haematol. 79 (1991), 256-262; McMillan et al., Blood 70 (1987), 1040 and Fujisawa et al., Blood 79 (1991); 1441). However, it is still difficult to characterize binding epitopes and to ascertain the pathogenic significance of the autoantibodies due to the limited quantity of autoantibodies which can be obtained from AITP patients. It has only been possible to obtain a few human monoclonal antibodies from lymphocytes of AITP patients which react with GPIIb/IIIa AIPT using the hybridoma technique (Kunicki et al., Hum. Antibodies Hybridiomas 1 (1990) 83-95).

Natural autoantibodies against various selfantigens, for example against intracellular and cytoskeletal

components of human platelets, have also been reported to occur in healthy individuals (Guilbert et al., J. Immunol. 128 (1982), 2779-2787; Hurez et al., Eur. J. Immunol. 23 (1993), 783-789 and Pfueller et al., Clin. Exp. Immunol. 79 (1990), 367-373). Some of these autoantibodies which have been observed in sera from healthy individuals can also be directed against platelet-membrane proteins (Souberbielle, Eur. J. Haematol. 56 (1996), 178-180). However, the role of these natural autoantibodies, and there relationship to disease-associated autoantibodies, is still unknown.

Corticosteroids can be used for treating AITP. About half of the patients react within 4 weeks to an administration of prednisone; however long-term remissions are only rarely seen. The administration of high doses of intravenous immunoglobulin (IVIgG) is recommended as an emergency treatment for patients who are exhibiting severe bleeding or extremely low platelet counts. This treatment is followed in most patients by a rapid, but usually only transient, increase in the platelet count. The mechanisms by which corticosteroids and IVIgG act in the treatment of AITP are still unknown. Investigations carried out by Berchtold et al., (Blood 74 (1989), 2414-2417 and Berchtold and Wenger, Blood 81 (1993), 1246-1250) have disclosed that antiiodiotypic antibodies which are present in IVIgG can inhibit the binding of autoantibodies to platelet glycoproteins.

The problem underlying the present application is that of identifying novel DNA sequences which are responsible for autoantibodies binding to GPIIb/IIIa. This approach can be used for making available novel pharmaceutical preparations which can be employed for improving the diagnosis and therapy of AITP.

It was surprisingly possible to identify binding sequences from autoantibodies after using peripheral circulating B cells from a healthy human donor to prepare a combinatorial phagemid display library of human antibody heavy and light chains. Following the presentation of human heavy and light antibody Fab fragments on the surface of the filamentous phage M13, it was possible to identify phage clones which exhibit specific binding to GPIIb/IIIa.

For this, the phagemid library was brought consecutively into contact with thrombasthenic platelets lacking GPIIb/IIIa (negative selection) and normal platelets (positive selection). After several rounds of selection and amplification by infecting E.coli, 23 clones were obtained which were able to bind to the GPIIb/IIIa complex. Inhibition studies using pools of monoclonal antibodies directed against the GPIIb/IIIa yielded two groups of clones: both groups were inhibited by monoclonal antibodies which were specific for the GPIIb/IIIa complex and one group was also inhibited by a GPIIb-specific monoclonal antibody. These findings were confirmed by carrying out a DNA analysis of the clones which indicated the presence of 2 different anti-GPIIb/IIIa phage clones. These results demonstrate that 2 GPIIb/IIIa-specific phage clones, i.e. autoantibodies, can be cloned from the genome of a healthy individual and that these clones are able to recognize confirmational epitopes belonging to the GPIIb/IIIa complex. Inhibition studies furthermore established that both phage clones inhibit the binding of platelet-associated autoantibodies from AITP patients to purified GPIIb/IIIa and therefore presumably recognize GPIIb/IIIa epitopes which are AITP-associated. Since the phage clones contain the antigen-binding sequences of natural autoantibodies which are derived from the genome of a healthy individual, this finding can lead to new insights into

the origin of platelet-associated autoantibodies in AITP.

In addition to this, it is possible to use the novel phage clones to produce recombinant antiidiotypic antibodies against anti-GPIIb/IIIa autoantibodies, with the anti-GPIIb/IIIa phage clones being used as antigen. The recombinant antiidiotypic antibodies which can be obtained in this way constitute an attractive clinical alternative to using IVIgG.

The nucleotide sequences of the identified phage clones, and the amino acid sequences which are deduced from these nucleotide sequences, are depicted in the sequencing listings SEQ ID No. 1 to 8 (autoantibodies) and SEQ ID No. 9 to 18 (antiidiotypic antibodies).

I. Autoantibodies

A first aspect of the present invention relates to nucleic acids which encode auto-antibodies. Part of the subject-matter of the invention is therefore a nucleic acid which encodes the heavy chain of a human antibody, or a functional derivative or a fragment thereof, and encompasses a CDR3 region, selected from:

(a) a nucleotide sequence which encodes the amino acid sequence:
V L P F D P I S M D V, (SEQ ID NO: 31) -(I)-

(b) a nucleotide sequence which encodes the amino acid sequence:
A L G S W G G W D H Y M D V, (SEQ ID NO: 32) -(II)-

(c) a nucleotide sequence which encodes an amino acid sequence having an homology of at least 80%, and preferably at least 90%, with an amino acid sequence from (a) or (b), and

(d) a nucleotide sequence which encodes an amino acid sequence having an equivalent ability to bind to GPIIb/IIIa.

The novel nucleic acid furthermore preferably comprises a CDR1 region selected from:

(a) a nucleotide sequence which encodes the amino acid sequence:
G Y S W R, (SEQ ID NO:33 ~~(III)~~)

(b) a nucleotide sequence which encodes the amino acid sequence:
S Y A M H, (SEQ ID NO: 34 ~~(IV)~~)
and

(c) a nucleotide sequence which encodes an amino acid sequence having an homology of at least 80%, and preferably at least 90%, with an amino acid sequence from (a) or (b).

The novel nucleic acid preferably furthermore comprises a CDR2 region selected from:

(a) a nucleotide sequence which encodes the amino acid sequence:
D I S Y S G S T K Y K P S L R S, (SEQ ID NO:35 ~~(V)~~)

(b) a nucleotide sequence which encodes the amino acid sequence:
V I S Y D G S N K Y Y A D S V K G, (SEQ ID NO: 36 ~~(VI)~~)
and

(c) a nucleotide sequence which encodes an amino acid sequence having an homology of at least 80%, and preferably of at least 90%, with an amino acid sequence from (a) or (b).

A second aspect of the present invention is a nucleic acid which encodes the light chain of a human antibody, or a functional derivative or a fragment thereof, and comprises a CDR3 region, selected from:

(a) a nucleotide sequence which encodes the amino acid sequence:
A T W D D G L N G P V, (SEQ ID NO: 37 ~~(VII)~~)

(b) a nucleotide sequence which encodes the amino acid sequence:

A A W D D S L N G W V, (SEQ ID NO: 38) ~~(VIII)~~

(c) a nucleotide sequence which encodes an amino acid sequence having an homology of at least 80%, and preferably of at least 90%, with an amino acid sequence from (a) or (b), and

(d) a nucleotide sequence which encodes an amino acid sequence having an equivalent ability to bind to GPIIb/IIIa.

The novel nucleic acid preferably furthermore comprises a CDR1 region selected from:

(a) a nucleotide sequence which encodes the amino acid sequence:
S G S S S N I R S N P V S, (SEQ ID NO: 39) ~~(IX)~~

(b) a nucleotide sequence which encodes the amino acid sequence:
S G S S S N I G S N T V N, (SEQ ID NO: 40) ~~(X)~~
and

(c) a nucleotide sequence which encodes an amino acid sequence having an homology of at least 80%, and preferably at least 90%, with an amino acid sequence from (a) or (b).

In addition, the novel nucleic acid preferably further comprises a CDR2 region selected from:

(a) a nucleotide sequence which encodes the amino acid sequence:
G S H Q R P S, (SEQ ID NO: 41) ~~(XI)~~

(b) a nucleotide sequence which encodes the amino acid sequence:
S N N Q R P S, (SEQ ID NO: 42) ~~(XII)~~
and

(c) a nucleotide sequence which encodes an amino acid sequence having an homology of at least 80%, and preferably at least 90%, with an amino acid sequence from (a) or (b).

II. Antiidiotypic antibodies

A second aspect of the present invention relates to nucleic acids which encode antiidiotypic antibodies. Part of the subject-matter of the invention is therefore a nucleic acid which encodes the heavy chain of a human antibody, or a functional derivative or a fragment thereof, and comprises a CDR3 region, selected from:

(a) a nucleotide sequence which encodes the amino acid sequence:
V R D L G Y R V L S T F T F D I, ~~(XIII)~~ (SEQ ID NO: 43)

(b) a nucleotide sequence which encodes the amino acid sequence:
D G R S G S Y A R F D G M D V, ~~(XIV)~~ (SEQ ID NO: 44)

(c) a nucleotide sequence which encodes the amino acid sequence:
M G S S V V A T Y N A F D I, ~~(XV)~~ (SEQ ID NO: 45)

(d) a nucleotide sequence which encodes the amino acid sequence:
D A D G D G F S P Y Y F P Y, ~~(XVI)~~ (SEQ ID NO: 46)

(e) a nucleotide sequence which encodes the amino acid sequence:
L R N D G W N D G F D I, ~~(XVII)~~ (SEQ ID NO: 47)

(f) a nucleotide sequence which encodes the amino acid sequence:
D S E T A I A A A G R F D I, ~~(XVIII)~~ (SEQ ID NO: 48)

(g) a nucleotide sequence which encodes the amino acid sequence:
E D G T T V P S Q P L E F, ~~(XIX)~~ (SEQ ID NO: 49)

(h) a nucleotide sequence which encodes the amino acid sequence:
G S G S Y L G Y Y F D Y, ~~(XX)~~ (SEQ ID NO: 50)

(i) a nucleotide sequence which encodes the amino acid sequence:
G L R S Y N Y G R N L D Y, ~~(XXI)~~ (SEQ ID NO: 51)

(j) a nucleotide sequence which encodes an amino acid sequence having an homology of at least

80%, and preferably of at least 90%, with an amino acid sequence from (a), (b), (c) or (d), and

(k) a nucleotide sequence which encodes an amino acid sequence having an equivalent ability to bind to autoantibodies against GPIIb/IIIa.

The novel nucleic acid furthermore preferably comprises a CDR1 region selected from: a nucleotide sequence which encodes the amino acid sequences N F A M S, S Y T M H, D Y A L H or S H Y W S shown in Tab. 7a, a nucleotide sequence which encodes the amino acid sequence T Y Y W S, a nucleotide sequence which encodes the amino acid sequences D Y G M H, S H T I S, K Y A I H or E L S M H shown in Tab. 7b, and a nucleotide sequence which encodes an amino acid sequence having an homology of at least 80%, and preferably at least 90%, with one of the previously mentioned amino acid sequences.

Preferably, the novel nucleic acid furthermore comprises a CDR2 region selected from a nucleotide sequence which encodes the amino acid sequences G I S G G G L L T H Y A (D/N) S V K G, L I S Y D G S N K Y Y A D S V K G, G I S W D S T S I G Y A D S V K G or F I Y D G A R T R F N P S L R S shown in Tab. 7a, a nucelotide sequence which encodes the amino acid sequence YIYYSGNTNYNPSLKS, a nucleotide sequence which encodes the amino acid sequences A I S Y D G S N K Y Y A D S V K G, G I T P I F G T V N Y A Q K F Q G, A I S S N G G N T Y Y A D S V K G or G F D P E D G E T I Y A Q K F Q G shown in Tab. 7b, and a nucleotide sequence which encodes an amino acid sequence having an homology of at least 80%, and preferably of at least 90%, with one of the previously mentioned amino acid sequences.

Another part of the subject-matter of the present invention is a nucleic acid which encodes the light

chain of a human antibody, or a functional derivative or a fragment thereof, and comprises a CDR3 region, selected from:

(a) a nucleotide sequence which encodes the amino acid sequence:
C S Y V H S S T N, (SEQ ID NO: 52) ~~(XXII)~~

(b) a nucleotide sequence which encodes the amino acid sequence:
Q V W D N T N D Q, (SEQ ID NO: 53) ~~(XXIII)~~

(c) a nucleotide sequence which encodes an amino acid sequence having an homology of at least 80%, and preferably at least 90%, with an amino acid sequence from (a), and

(d) a nucleotide sequence which encodes an amino acid sequence having an equivalent ability to bind to autoantibodies against GPIIb/IIIa.

Preferably, the novel nucleic acid furthermore comprises a CDR1 region selected from a nucleotide sequence which encodes the amino acid sequence T G T S S A I G N Y N F V P (SEQ ID NO: 66) shown in Tab. 7a, a nucleotide sequence which encodes the amino acid sequence G G Y K I G S K S V H (SEQ ID NO: 67) shown in Tab. 7b, and a nucleotide sequence which encodes an amino acid sequence having an homology of at least 80%, and preferably of at least 90%, with the previously mentioned amino acid sequence.

In addition, the novel nucleic acid preferably furthermore comprises a CDR2 region selected from a nucleotide sequence which encodes the amino acid sequence E G S K R P S (SEQ ID NO: 68) shown in Tab. 7a, a nucleotide sequence which encodes the amino acid sequence E D S Y R P S (SEQ ID NO: 69) shown in Tab. 7b, and a nucleotide sequence which encodes an amino acid sequence having an homology of at least 80%, and preferably at least 90%, with the previously mentioned amino acid sequence.

Within the meaning of the present invention, the phrase "functional derivative of a chain of a human antibody" is to be understood as meaning a polypeptide which encompasses at least a CDR3 region of the heavy and/or light chain, as defined above, and which is able, where appropriate together with the relevant complementary chain of the human antibody (or a derivative of such a chain), to form an antibody derivative which possesses a recognition specificity for an antigen which is equivalent to that possessed by the non-derivatized antibody. Preferably, such an antibody derivative has a binding constant for the relevant antigen of at least 10^{-6} 1/mol, preferably of at least 10^{-3} 1/mol.

Functional derivatives of chains of a human antibody can be prepared, for example, by using recombinant DNA techniques to delete, substitute and/or insert segments of the gene encoding the relevant polypeptide.

Single-chain antibodies, which can, for example, be composed of the variable domains of the H and L chains or one or two H chain domains and, where appropriate a constant domain, are particularly preferred functional derivatives of antibody chains or antibodies. The preparation of such constructs is described in Hoogenboom et al., Immunol. Rev. 130 (1992), 41-68; Barbas III, Methods: Companion Methods Enzymol. 2 (1991), 119 and Plückthun, Immunochemistry (1994), Marcel Dekker Inc., Chapter 9, 210-235.

Within the meaning of the present invention, the phrase "equivalent ability to bind" is to be understood as being a binding affinity and/or specificity, i.e. epitope recognition, which is the same as that in the specifically disclosed sequences.

Another part of the subject-matter of the present invention is a vector which contains at least one copy

of a novel nucleic acid. This vector can be a prokaryotic vector or a eukaryotic vector. Plasmids, cosmids and bacteriophages are examples of prokaryotic vectors. Such vectors are, for example, described in detail in Chapters 1 to 4 in Sambrook et al., Molecular Cloning. A Laboratory Manual, 2nd edition (1989), Cold Spring Harbor Laboratory Press. A prokaryotic vector is preferably a plasmid or a phage.

On the other hand, the vector can also be a eukaryotic vector, e.g. a yeast vector, an insect vector (baculovirus) or a mammalian vector (plasmid vector or viral vector). Examples of eukaryotic vectors are described in Sambrook at al., loc. cit., Chapter 16, and Winnacker, Gene und Klone, Eine Einführung für die Gentechnologie [Genes and clones, an introduction to genetic engineering] (1985), VCH Verlagsgesellschaft, in particular Chapters 5, 8 and 10.

Yet another part of the subject-matter of the present invention is a cell which expresses a novel nucleic acid, or a cell which is transformed with a novel nucleic acid or with a novel vector. The cell can be a prokaryotic cell (e.g. a Gram-negative bacterial cell, in particular E.coli) or a eukaryotic cell (e.g. a yeast, plant or mammalian cell). Examples of suitable cells and methods for introducing the novel nucleic acid into such cells can be found in the above literature references.

Another part of the subject-matter of the present invention is a polypeptide which is encoded by a novel nucleic acid, in particular a recombinant polypeptide. Particularly preferably, the polypeptide contains the variable domain of the H chain and/or L chain of a human antibody.

Particular preference is given to a polypeptide which exhibits antibody properties and whose subunit components are a heavy chain, or a functional derivative thereof, and a light chain, or a functional derivative thereof. The polypeptide can be composed of two separate chains or be present as a single-chain polypeptide.

Yet another part of the subject-matter of the present invention is an antibody against a novel polypeptide, which antibody is directed against a region of the polypeptide which is responsible for recognizing the antigen. This antibody can be a polyclonal antiserum, a monoclonal antibody or a fragment of a polyclonal or monoclonal antibody (e.g. a Fab, $F(ab)_2$, Fab' or $F(ab')_2$ fragment). The antibody is preferably directed against the CDR3 region of the heavy and/or light antibody chain of the novel polypeptide, or a region thereof. Known methods can be used to obtain such antibodies by immunizing an experimental animal with a peptide or polypeptide which contains a novel CDR3 region and isolating the resulting polyclonal antibody from the experimental animal. In addition, monoclonal antibodies can be obtained by fusing an antibody-producing B cell from the experimental animal with a leukaemia cell in accordance with the method of Köhler and Milstein or a further development of this method. In addition, recombinant antibodies which are directed against the CDR3 region of the novel polypeptide can also be obtained by screening a suitable phagemid library, e.g. a phagemid library from a healthy human donor, with a novel polypeptide being used as the antigen.

The invention also relates to a pharmaceutical composition which comprises a nucleic acid, a vector, a polypeptide, an antibody or a cell as previously mentioned, as active component, where appropriate together with other active components and also

pharmaceutically customary adjuvants, additives or excipients.

The pharmaceutical composition can be used for preparing a diagnostic or therapeutic agent. Examples of diagnostic uses are the diagnosis of AITP or of a predisposition for AITP. Another preferred diagnostic use is that of monitoring the course of the AITP disease.

The use of the pharmaceutical composition as a diagnostic agent can comprise, for example, detecting a B cell subpopulation which is expressing a novel polypeptide as the antibody. This antibody can be detected, for example, at the nucleic acid level, e.g. by means of a nucleic-acid-hybridization assay, together with prior amplification where appropriate. On the other hand, the antibody can also be detected as to the protein level by means of an immunoassay using antigens or antibodies which react specifically with the polypeptide.

Furthermore, the novel pharmaceutical composition can also be applied in the therapeutic field, in particular for the prevention or therapy of AITP. This therapeutic use can, for example, be based on stimulating the production of anti-autoantibodies. For this, the novel autoantibody polypeptide can, for example, be administered to a patient, thereby eliciting and/or stimulating the formation of antiidiotypic antibodies. In this connection, this administration can be effected in accordance with customary immunization protocols (Fox et al., J. Pharmacol. Exp. Ther. 279 (1996), 1000-1008; Whittum-Hudson et al., Nat. Med. 2 (1996), 1116-1121; Jardieu, Curr. Opin. Immunol. 7 (1995), 779-782). On the other hand, the expression of antibody genes can be inhibited specifically by administering suitable antisense nucleic acids. The novel

antiidiotypic antibody polypeptide can be administered to a patient in order to achieve direct inhibition of the autoantibody activity.

Investigations carried out on the novel autoantibody polypeptides have shown that these polypeptides are surprisingly able to inhibit the binding of fibrinogen to blood platelets. The novel autoantibody polypeptides and antiidiotypic antibody polypeptides can therefore be employed, where appropriate in combination, as agents for modulating blood coagulation, in particular for preventing a thrombosis, for example following cardiac infarctions or strokes, or in association with venous thromboses together with lung embolisms or ischaemias, etc.

Murine monoclonal antibodies, e.g. the monoclonal antibody 7E3 (cf., e.g., US patent 5,440,020) or fragments thereof (e.g. the commercially available Fab fragment ReoPro®), or short synthetic peptides, have hitherto been used as fibrinogen antagonists for therapeutic purposes. However, murine monoclonal antibodies and antibody fragments suffer from the disadvantage that, as a result of their immunogenicity, they give rise to undesirable side reactions when used for treating human patients, while short peptides are generally degraded very rapidly. As compared with these known agents, the novel polypeptides have the advantage that they consist of amino acid sequences of human origin and therefore exhibit fewer undesirable side effects than do corresponding murine antibodies or antibody fragments, and that, because of their size, they are not subjected to such rapid degradation as are peptides.

The invention therefore relates to the use of a novel nucleic acid, in particular a nucleic acid which encodes an autoantibody polypeptide, of a vector which

contains this nucleic acid, of a cell which is transformed with the nucleic acid or the vector, of a polypeptide which is encoded by the nucleic acid, or of a pharmaceutical composition which comprises one or more of the said substances, for preparing an agent for affecting and in particular inhibiting the binding of fibrinogen to blood platelets. Preference is given to using the agent for modulating blood coagulation, in particular for dissolving thrombi and/or for preventing the formation of thrombi. The administration of the novel pharmaceutical composition can be effected in accordance with protocols which have already been established for murine antibodies or antibody fragments.

Yet another part of the subject-matter of the invention is a process for isolating phagemid clones which express nucleic acids which encode autoantibodies against GPIIb/IIIa or encode antiidiotypic antibodies which are directed against these autoantibodies, characterized in that a phagemid library is prepared from lymphocytes from a human donor and the desired phagemid clones are isolated by affinity selection, comprising negative and positive selection steps. Preferably, the process also involves isolating antibody-encoding nucleic acids from the clones and/or using the antibody-encoding nucleic acids for expressing recombinant antibody chains or derivatives or fragments thereof.

The invention is also explained by the following examples, figures and sequence listings, in which

SEQ ID No. 1 shows the nucleotide sequence of the H chain of a novel antibody (phagemid clone PDG7), with framework region (FR)1 extending from bp 1 to 90, complement-determining region (CDR)1 from bp 91 to

105, FR2 from bp 106 to 147, CDR2 from bp 148 to 195, FR3 from bp 196 to 291, CDR3 from bp 292 to 324 and FR4 from bp 325 to 357,

SEQ ID No.2 shows the amino acid sequence corresponding to the nucleotide sequence depicted in SEQ ID No.1, with FR1 extending from AA 1 to 30, CDR1 from AA 31 to 35, FR2 from AA 36 to 49, CDR2 from AA 50 to 65, FR3 from AA 66 to 97, CDR3 from AA 98 to 108 and FR4 from AA 109 to 119,

SEQ ID No.3 shows the nucleotide sequence of the L chain of a novel polypeptide (phagemid clone PDG7), with FR1 extending from bp 1 to 60, CDR1 from bp 61 to 99, FR2 from bp 100 to 144, CDR2 from bp 145 to 165, FR3 from bp 166 to 261, CDR3 from bp 262 to 294 and FR4 from bp 295 to 333,

SEQ ID No.4 shows the amino acid sequence corresponding to the nucleotide sequence given in SEQ ID No. 3, with FR1 extending from AA 1 to 20, CDR1 from AA 21 to 33, FR2 from AA 34 to 48, CDR2 from AA 49 to 55, FR3 from AA 56 to 87, CDR3 from AA 88 to 98 and FR4 from AA 99 to 11 [sic],

SEQ ID No.5 shows the nucleotide sequence of the H chain of a novel polypeptide (phagemid clone PDG13), with FR1 extending from bp 1 to 90, CDR1 from bp 91 to 109, FR2 from bp 106 to 147, CDR2 from bp 148 to 198, FR3 from bp 199 to 294, CDR3 from

bp 295 to 336 and FR4 from bp 337 to 369,

SEQ ID No.6 shows the amino sequence corresponding to the nucleotide sequence depicted in SEQ ID No.5, with FR1 extending from AA 1 to 30, CDR1 from AA 31 to 35, FR2 from AA 36 to 49, CD2 from AA 50 to 66, FR3 from AA 67 to 98, CDR3 from AA 99 to 112 and FR4 from AA 113 to 123,

SEQ ID No.7 shows the nucleotide sequence of the L chain of a novel polypeptide (phagemid clone PGD13), with FR1 extending from bp 1 to 60, CDR1 from bp 61 to 99, FR2 from bp 100 to 144, CDR2 from bp 145 to 165, FR3 from bp 166 to 261, CDR3 from bp 262 to 294 and FR4 from bp 295 to 333,

SEQ ID No.8 shows the amino acid sequence of the nucleotide sequence depicted in SEQ ID No. 7, with FR1 extending from AA 1 to 20, CDR1 from AA 21 to 33, FR2 from AA 34 to 48, CDR2 from AA 49 to 55, FR3 from AA 56 to 87, CDR3 from AA 88 to 98 and FR4 from AA 99 to 111,

SEQ ID No.9 shows the nucleotide sequence of the H chain of a novel polypeptide (phagemid clone AI-X16), with FR1 extending from bp 1 to 90, CDR1 from bp 91 to 105, FR2 from bp 106 to 147, CDR2 from bp 148 to 198, FR3 from bp 199 to 288, CDR3 from bp 289 to 336 and FR4 from bp 337 to 369,

SEQ ID No.10 shows the amino acid sequence of the nucleotide sequence depicted in SEQ ID No. 9, with FR1 extending from AA 1 to 30, CDR1 from AA 31 to 35, FR2 from AA 36 to 49, CDR2 from AA 50 to 66, FR3 from AA 67 to 96, CDR3 from AA 97 to 112 and FR4 from AA 113 to 123,

SEQ ID No. 11 shows the nucleotide sequence of the L chain of a novel polypeptide (phagemid clone AI-X16), with FR1 extending from bp 1 to 60, CDR1 from bp 61 to 102, FR2 from bp 103 to 147, CDR2 from bp 148 to 168, FR3 from bp 169 to 264, CDR3 from [lacuna] 265 to 291 and FR4 from bp 292 to 375,

SEQ ID No. 12 shows the amino acid sequence of the nucleotide sequence depicted in SEQ ID No. 11, with FR1 extending from AA 1 to 20, CDR1 from AA 21 to 34, FR2 from AA 35 to 49, CDR2 from AA 50 to 56, FR3 from AA 57 to 88, CDR3 from AA 89 to 97 and FR4 from AA 89 to 125,

SEQ ID No. 13 shows the nucleotide sequence of the H chain of a novel polypeptide (phagemid clone AI-X20), with FR1 extending from bp 1 to 90, CDR1 from bp 91 to 105, FR2 from bp 106 to 147, CDR2 from bp 148 to 195, FR3 from bp 196 to 291, CDR3 from bp 292 to 333 and FR4 from bp 334 to 366,

SEQ ID No. 14 shows the amino acid sequence of the nucleotide sequence depicted in SEQ ID No. 13, with FR1 extending from AA 1 to 30, CDR1 from AA 31 to 35, FR2 from AA

36 to 49, CDR2 from AA 50 to 65, FR3 from AA 66 to 97, CDR3 from AA 98 to 111 and FR4 from AA 112 to 122,

SEQ ID No. 15 shows the nucleotide sequence of the H chain of a novel polypeptide (phagemid clone AI-X39), with FR extending from bp 1 to 90, CDR1 from bp 91 to 105, FR2 from bp 106 to 147, CDR2 from pb [sic] 148 to 198, FR3 from bp 199 to 294, CDR3 from bp 295 to 339 and FR4 from 340 to 372,

SEQ ID No. 16 shows the amino acid sequence of the nucleotide sequence depicted in SEQ ID No. 15, with FR1 extending from AA 1 to 30, CDR1 from AA 31 to 35, FR2 from AA 36 to 49, CDR2 from AA 50 to 66, FR3 from AA 67 to 98, CDR3 from AA 99 to 113 and FR 4 from AA 114 to 124,

SEQ ID No. 17 shows the nucleotide sequence of the H chain of a novel polypeptide (phagemid clone AI-X40), with FR1 extending from bp 1 to 90, CDR1 from bp 91 to 105, FR2 from bp 106 to 147, CDR2 from bp 148 to 198, FR3 from bp 199 to 297, CDR3 from bp 298 to 339 and FR4 from bp 340 to 372,

SEQ ID No. 18 shows the amino acid sequence of the nucleotide sequence depicted in SEQ ID No. 17, with FR1 extending from AA 1 to 30, CDR1 from AA 31 to 35, FR2 from AA 36 to 49, CDR2 from AA 50 to 66, FR3 from AA 67 to 99, CDR3 from AA 100 to 113 and FR4 from AA 114 to 124,

SEQ ID No. 19 shows the nucleotide sequence of the H chain of a novel polypeptide (phagemid clone AI-X2), with FR1 extending from bp 1 to 90, CDR1 from bp 91 to 105, FR2 from bp 106 to 147, CDR2 from bp 148 to 195, FR3 from bp 196 to 291, CDR3 from bp 292 to 327 and FR4 from bp 328 to 360,

SEQ ID No. 20 shows the amino acid sequence of the nucleotide sequence depicted in SEQ ID No. 19, with FR1 extending from AA 1 to 30, CDR1 from AA 31 to 35, FR2 from AA 36 to 49, CDR2 from AA 50 to 65, FR3 from AA 66 to 97, CDR3 from AA 98 to 109 and FR4 from AA 110 to 120,

SEQ ID No. 21 shows the nucleotide sequence of the H chain of a novel polypeptide (phagemid clone AI-B14), with FR1 extending from bp 1 to 90, CDR1 from bp 91 to 105, FR2 from bp 106 to 147, CDR2 from bp 148 to 198, FR3 from bp 199 to 294, CDR3 from bp 295 to 336 and FR4 from bp 337 to 369;

The following variations in the sequence were also found: a C can be present at position 7, while a G can be present at position 9, a G at position 13, a G at position 15, an A at position 91, a G at position 92, a C at position 98, a T at position 149, an A at position 205, an A at position 228, an A at position 251, a T at position 253 and/or an A at position 284. The consequence of this is that, in the amino acid sequence (cf. SEQ ID No. 22), a Q can be present at

position 3, while a V can be present at position 5, an S at position 31, an A at position 33, a V at position 50, a T at position 69, a K at position 76, an N at position 84, an S at position 85 and/or a Y at position 95.

SEQ ID No. 22 shows the amino acid sequence of the nucleotide sequence depicted in SEQ ID No. 21, with FR1 extending from AA 1 to 30, CDR1 from AA 31 to 35, FR2 from AA 36 to 49, CDR2 from AA 50 to 66, FR3 from AA 67 to 98, CDR3 from AA 99 to 112 and FR4 from AA 113 to 123,

SEQ ID No. 23 shows the nucleotide sequence of the H chain of a novel polypeptide (phagemid clone AI-B18), with FR1 extending from bp 1 to 90, CDR1 from bp 91 to 105, FR2 from bp 106 to 147, CDR2 from bp 148 to 198, FR3 from bp 199 to 294, CDR3 from bp 295 to 333 and FR4 from bp 334 to 366;

The following variations in the nucleotide sequence were also found: thus, a C can be present at position 7, while a G can be present at position 13, a C at position 16, an A at position 56, a T at position 94, a G at position 97, a T at position 155, a C at position 173, a T at position 223, a T or a C at position 252, a G at position 261, a G at position 267, an A at position 271, a C at position 275 and/or a G at position 277. The consequence of this is that, in the corresponding amino acid sequence (cf. SEQ ID No. 24), a Q can be present

at position 3, while a V can be present at position 5, a Q at position 6, a K at position 19, a Y at position 32, an A at position 33, an I at position 52, an A at position 58, an S at position 75, an S at position 84, an R at position 87, an E at position 89, a T at position 91, an A at position 92 and/or a V at position 93.

SEQ ID No. 24 shows the amino acid sequence of the nucleotide sequence depicted in SEQ ID No. 23, with FR1 extending from AA 1 to 30, CDR1 from AA 31 to 35, FR2 from AA 36 to 49, CDR2 from AA 50 to 66, FR3 from AA 67 to 98, CDR2 from AA 99 to 111 and FR4 from AA 112 to 122,

SEQ ID No. 25 shows the nucleotide sequence of the H chain of a novel polypeptide (phagemid clone AI-B24), with FR1 extending from bp 1 to 90, CDR1 from bp 91 to 105, FR2 from bp 106 to 147, CDR2 from bp 148 to 198, FR3 from bp 199 to 294, CDR3 from bp 295 to 330 and FR4 from bp 331 to 363;

The following variations in the nucleotide sequence were also found: a C can be present at position 7, while a G can be present at position 9, a G at position 13, a G at position 15, a G at position 31, an A at position 46, a G at position 67, a G at position 89, a G at position 92, a C at position 93, a G at position 98, a G at position 102, a G at position 140, a G at position 141, a G at position 145, a T at position 149, a

T at position 157, an A at position 158, a G at position 160, an A at position 166, an A at position 173, a T at position 235, an A at position 251, a C at position 290 and/or an A at position 293. The consequence of this is that, in the corresponding amino acid sequence (cf. SEQ ID No. 26), a Q can be present at position 3, while a V can be present at position 5, a V at position 11, an R at position 16, an A at position 23, an S at position 30, an S at position 31, a G at position 33, an M at position 34, a W at position 47, an A at position 49, a V at position 50, a Y at position 53, a D at position 54, an S at position 56, a K at position 58, an L at position 79, an N at position 84, an A at position 97 and/or a K at position 98.

SEQ ID No. 26 shows the amino acid sequence of the nucleotide sequence depicted in SEQ ID No. 25, with FR1 extending from AA 1 to 30, CDR1 from AA 31 to 35, FR2 from AA 36 to 49, CDR2 from AA 50 to 66, FR3 from AA 67 to 98, CDR3 from AA 99 to 110 and FR4 from AA 111 to 121,

SEQ ID No. 27 shows the nucleotide sequence of the L chain of a novel polypeptide (phagemid clone AI-B24), with FR1 extending from bp 1 to 60, CDR1 from bp 61 to 96, FR2 from bp 97 to 138, CDR2 from bp 139 to 159, FR3 from bp 160 to 255, CDR3 from bp 256 to 282 and FR4 from bp 283 to 366;

The following variations in the nucleotide sequence were also found: a C or a T can be present at position 4, while a G can be present at position 37, an A at position 40, a G at position 50, an A at position 67, a T at position 72, an A at position 133, a T at position 136, a T or a C at position 138, a G at position 148, a T at position 160, a T at position 161, a T or a C at position 162, a C at position 200, a T at position 217, a G at position 218, an A or a C at position 220, a G at position 269, a T at position 271, a G at position 272, a G at position 275 and/or a T or a C at position 282. The consequence of this is that, in the corresponding amino acid sequence (cf. SEQ ID No. 28), an I can be present at position 2, while a G can be present at position 13, a K at position 14, an R at position 17, an N at position 23, an N at position 24, an I at position 45, a Y at position 47, a D at position 50, an F at position 54, a T at position 67, an S at position 73, an R at position 74, an S at position 90, an S at position 91, an S at position 92 and/or an H at position 94.

SEQ ID No. 28 shows the amino acid sequence of the nucleotide sequence depicted in SEQ ID No. 27, with FR1 extending from AA 1 to 20, CDR1 from AA 21 to 32, FR2 from AA 33 to 46, CDR2 from AA 47 to 53, FR3 from AA 54 to 85, CDR3 from AA 86 to 94 and FR4 from AA 95 to 122,

SEQ ID No. 29 shows the nucleotide sequence of the H chain of a novel polypeptide (phagemid clone AI-B38), with FR1 extending from bp 1 to 90, CDR1 from bp 91 to 105, FR2 from bp 106 to 147, CDR2 from bp 148 to 198, FR3 from bp 199 to 294, CDR3 from bp 295 to 333 and FR4 from bp 334 to 366;

The following variations in the nucleotide sequence were also found: a C can be present at position 7, while a G can be present at position 9, a G at position 13, an A at position 15 and/or a C at position 16. The consequence of this is that, in the corresponding amino acid sequence, a Q can be present at position 3, while a V can be present at position 5 and/or a Q can be present at position 6, and

SEQ ID No. 30 shows the amino acid sequence of the nucleotide sequence depicted in SEQ ID No. 29, with FR1 extending from AA 1 to 30, CDR1 from AA 31 to 35, FR2 from AA 36 to 49, CDR2 from AA 50 to 66, FR3 from AA 67 to 98, CDR3 from AA 99 to 111 and FR4 from AA 112 to 122.

Figure 1 shows the inhibition of the binding of autoantibody phabs (PDG-X) to GPIIb/IIIa which is brought about by adding the antiidiotypic antibody phab AI-X17.

Figure 2 shows the inhibition of the binding of autoantibody phabs (PDG-B) to blood platelets which is brought about by antiidiotypic antibody phabs AI-B,

Figure 3 shows the binding of autoantibody phabs to untreated and EDTA-treated blood platelets,

Figure 4 shows the inhibition of the binding of fibrinogen to GPIIb/IIIa which is brought about by autoantibody phabs,

Figures 5-7 show the inhibition of the binding of autoantibody phabs to GPIIb/IIIa which is brought about by the antibody 7E3 and the antibody fragment ReoPro®.

Examples

1. Identification of autoantibody sequences

1.1. Isolation of autoantibodies

Autoantibodies were obtained from 12 AITP patients (8 suffering from primary AITP, 3 suffering from AITP associated with SLE, 1 suffering from AITP associated with Sjögren's syndrome) by incubating patient plasma with purified GPIIb/IIIa at 4°C overnight and subsequently eluting, at room temperature for 15 min, in 0.2 mol/l glycine and 0.15 mol/l NaCl, pH 2.5. After centrifuging at 100,000 g for 30 min, the supernatant was neutralized with 1 mol/l Tris-HCl and dialysed overnight against Tris-buffered salt solution (TBS).

At the time of plasma withdrawal, all the patients were thrombocytopenic (platelet count < 150 × 10^9/l) and had normal or enlarged megakaryocytes in the bone marrow and were free of other detectable forms of immunothrombocytopenia.

1.2. Isolation of purified antigens

The antigens used were purified GPIIb/IIIa, a cytoplasmic fragment of GPIIIa (amino acids 721-744) and an extracellular fragment of GPIIIa (amino acids 468-690) (Beardsley, Blut 59 (1989), 47-51 and Phillips et al., Methods Enzymol. 215 (1992), 244-263).

1.3. Isolation of platelets for panning and immunoblotting

Platelet-enriched plasma was prepared by differential centrifugation from EDTA-anticoagulated blood samples taken from healthy human donors. The platelets were isolated by centrifuging at 2000 g for 15 min, then washed six times in citric acid buffer (pH 6.2) containing 50 mmol/l sodium citrate, 100 mmol/l NaCl and 125 mmol/l glucose, and finally resuspended in the same buffer.

The same enrichment protocol was used to obtain thrombasthenic platelets from a 14-year-old boy suffering from Glanzmann's type I thrombasthenia.

1.4. Monoclonal antibodies

Use was made of murine monoclonal antibodies which recognize the complexed form of GPIIb/IIIa and of antibodies which recognize GPIIb or GPIIIa selectively. These antibodies were isolated by means of customary immunization protocols using the corresponding antigens and are not AITP-associated. The isolation of such antibodies is described in Kouns et al. (J. Biol. Chem. 267 (1992), 18844-18851), Steiner et al. (Biochim. Biophys. Acta 1119 (1992), 12-21) and Häring et al. (Proc. Natl. Acad. Sci. USA 82 (1985), 4837-4841).

1.5. Phagemid library

A combinatorial Fab library was prepared in accordance with the method described by Vogel et al. (Eur. J. Immunol. 24 (1994), 1200-1207) using peripheral blood lymphocytes obtained from a healthy, preimmunized human donor. All the enzymes and oligonucleotides were obtained from Boehringer Mannheim GmbH (Mannheim, Germany) apart from the Taq polymerase (Perkin Elmer, NJ, USA). The primers for amplifying the H and L chains of the Fab molecules by PCR, the VCSM13 helper phage, and the Escherichia coli strain XL-Blue were obtained from Stratacyte (La Jolla, CA, USA). The phagemid pComb3 was obtained from Scripps Research Institute (La Jolla, CA, USA). The cloning, the transformation into XL-Blue cells and the preparation of phabs were carried out as described by Barbas III and Lerner, Methods: Companion Methods Enzymol. 2 (1991), 119). The phabs were precipitated with 4% (w/v) polyethylene glycol 8000 and 3% (w/v) NaCl and resuspended in PBS, pH 7.4. The resulting expression library contains 1×10^7 specificities.

1.6. Isolation of GPIIb/IIIa-specific phabs

GPIIb/IIIa-specific phabs were prepared by means of a total of 5 rounds of an affinity selection ("panning"). Following preabsorption (negative selection) with 5×10^7 thrombasthenic platelets, the phabs were incubated for 45 min with 10^8 normal platelets (positive selection). Bound phabs were then eluted with 0.05 mol/l sodium citrate, pH 2.5, and neutralized with 1 mol/l Tris buffer. After each round of panning, the enrichment of GPIIb/IIIa-specific phabs was monitored by titrating the phage-colony-forming units. After five rounds of selection, the eluted phabs were found to have been enriched by a factor of more than 100.

The pool of phabs obtained after the fourth round of selection was analysed more closely for its GPIIb/IIIa specificity. For this, 40 phab clones were selected at random and their binding specificity was ascertained in an immunodot assay. One µl of normal and thrombasthenic platelets (10^9 ml) [sic], and also purified GPIIb/IIIa (500 µg/ml), were added as drops onto nitrocellulose strips (Millipore Corporation, Bedford, MA, USA). The strips were blocked in TBS containing 0.15% casein (TBS-casein) and then incubated overnight together with the phabs, which had been diluted in TBS-casein. After three washes with TBS-0.1% Tween 20 (TBS-Tween), the bound phabs were detected with 4-chloro-1-α-naphthol (Merck, Darmstadt, Germany) following incubation with horseradish peroxidase-conjugated polyclonal rabbit anti-phage antibody (Vogel et al., loc. cit.) which had been diluted 1:1000 in TBS-casein.

The binding of phabs to platelets and purified GPIIb/IIIa was also tested after denaturing the proteins by heating (70°C) or by acid treatment (pH 2 with 0.5 N HCl) before dropping.

Of the 40 randomly selected clones, 23 (57.5%) reacted with GPIIb/IIIa, whereas 17 did not exhibit any binding. No binding of anti-GPIIb/IIIa [sic] to phabs was observed after denaturing the antigen by heat or pH 2 prior to the incubation, thereby demonstrating that intact GPIIb/IIIa is required for the phab binding. Determining the presence of Fab in negative phabs revealed that 15 of the clones (88%) did not contain any Fab molecules. The two Fab-positive clones which did not bind to GPIIb/IIIa could have a low binding affinity for GPIIb/IIIa.

1.7. Fab analysis

In order to test the positive phabs for kappa (κ), lambda (λ) and Fd chains, the anti-GPIIb/IIIa phabs were added as drops to nitrocellulose. The filters were incubated for 4 hours with peroxidase-labelled mouse anti-human λ, κ (The Binding Site Limited, Birmingham, England) and Fd antibodies (from the HP6045 myeloma cell line, ATCC1757, Rockville, MD, USA), which antibodies had been diluted 1:1000 in TBS-casein, and then developed by chemiluminescence (ECL, Amersham, Switzerland, Zurich, Switzerland). Testing 15 randomly selected anti-GPIIb/IIIa Fab clones for κ, λ and Fd chains showed that an Fd chain was present in 12 of the clones (80%) while the λ chain was present in all the clones.

Fab binding to GPIIb/IIIa on platelets was determined quantitatively by preincubating pool phabs with platelets at various concentrations. The supernatant was then analysed by an immunodot method. In this connection, it was established that from 1 to 3 × 10^4 phabs bind per platelet. This indicates that approximately 10 to 50% of the GPIIb/IIIa molecules per platelet can be occupied by phabs.

1.8. Characterizing the phab-binding epitopes

The epitope specificity of the phabs was determined by carrying out an inhibition test using a variety of monoclonal antibodies (see item 4 [sic]). 1 µl of thawed normal and thrombasthenic platelets (10^9/ml), purified GPIIb/IIIa (500 µg/ml), a peptide fragment of GPIIIa (amino acids 468-690, 500 µg/ml) and the cytoplasmic segment of GPIIb/IIIa (500 µg/ml) were in each case added as drops, in duplicate, onto nitrocellulose strips. After blocking, the phab clones (0.4 µg/ml Fab) were incubated overnight with or

without monoclonal antibody (1 μg/ml). The bound phabs were detected using peroxidase-labelled anti-phage antibody and 4-chloro-1-α-naphthol.

Two groups of phab clones were identified in these investigations. While Group A (5 clones) was inhibited moderately by a pool of all the antibodies, it was inhibited strongly by GPIIb/IIIa complex-specific antibodies. Anti-GPIIb antibodies had no effect. While Group B (10 clones) was inhibited completely by the pool of all the antibodies, it was inhibited to a lesser extent by the complex-specific antibody and also by the IIb-specific antibody. No group exhibited any reaction with GPIIIa-specific antibodies. The same results were obtained using either platelets or purified GPIIb/IIIa as the antigen. No phab binding to the cytoplasmic peptide or to the extracellular fragment of GPIIIa was found to occur.

A summary of these results is shown in Table 1.

Table 1

Inhibition of phab binding (mean value ± SD in %)				
Pools of monoclonal antibodies for inhibition	Group A phab clones (n = 5)		Group B phab clones (n = 10)	
	Platelets	Purified GPIIb/IIIa	Platelets	Purified GPIIb/IIIa
(1) Anti-GPIIB	0	0	49.1 ± 5.9	49.4 ± 9.2
(2) Anti-GPIIIa	0	0	0	0
(3) Anti-GPIIb/IIIa complex	77.8 ± 2.9	43.6 ± 2.1	58.6 ± 4.4	45.5 ± 8.0
Pool of all the antibodies (1)-(3)	47.6 ± 7.7	33.0 ± 10.8	95.9 ± 2.7	97.5 ± 7.5

1.9. Inhibition assays

The blocking, by the anti-GPIIb/IIIa phabs which had been found, of the binding of patient autoantibodies to GPIIb/IIIa was determined by means of inhibition assays. Two of the phab clones which had been identified as previously described (PDG16 and PDG31) were used for this purpose.

Serial dilutions of the eluted patient autoantibodies of from 1:3 to 1:1000 were analysed for binding to purified GPIIb/IIIa. This was done by performing an immunodot assay. 100 ng of purified GPIIb/IIIa were in each case added as drops, in triplicate, onto nitrocellulose strips and the filters were then blocked with TBS-casein. In order to block the binding of AITP autoantibodies to GPIIb/IIIa with phabs, the strips were incubated with 10^{11} phabs for 1 h and then incubated with varying dilutions of AITP autoantibodies for 4 h. Bound autoantibodies were detected using peroxidase-labelled anti-human IgG-Fc antibodies and ECL.

Anti-GPIIb/IIIa phabs inhibited the binding of autoantibodies obtained from 8 AITP patients. The inhibition range [sic] was [sic] from 10 to 46%, from 32 to 60% and from 20 to 67% for PTG16, PTG31 and the pool of the two phabs, respectively. These phabs had no effect on the binding of autoantibodies obtained from 4 AITP patients. Both groups contained autoantibodies derived from patients suffering from primary AITP and from disease-associated AITP.

The results which were obtained are summarized in Table 2.

Table 2

	Inhibition of the binding to purified GPIIb/IIIa by (%)		
AITP patient	**Phab clone PDG16**	**Phab clone PDG31**	**Pool of the two phab clones**
WS16	13	19	40
WS37	14	20	36
KC	24	22	28
KK	22	22	40
KP	10	36	60
WS2	25	55	65
KS	60	56	64
KL	0	15	10
KG	0	0	0
KM	0	0	0
KE	0	0	0
KR	0	0	0

1.10 DNA sequence analysis

Plasmid DNA was purified from four Group A phab clones and 4 group [lacuna] clones using the Nukleobond® AX PC 20 purification kit (Macherey-Nagel AG, Oensingen, Switzerland).

The nucleic acid sequencing was carried out on an ABI373A sequencing system using a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit. The primers were obtained from Microsynth, Balgach, Switzerland. The following primers were used for sequencing the H chain: Chγ1 (5'-CGC TGT GCC CCC AGA GGT-3') and PCH (5'-GGC CGC AAA TTC TAT TTC AAG G-3'). The following primers were used for sequencing the L chain: Cλ (5'-GAG ACA CAC CAG TGT GGC-3'), Ck (5'-CAC AAC AGA GGC AGT TCC-3') and PCL (5'-CTA AAC TAG CTA GTC TCC-3'). The amino acid sequences which were deduced from the DNA

sequence were compared with GenEMBL-Genbank and strain lines were assigned to VH and Vλ families.

The VH and Vλ nucleotide sequences of the 4 phab clones from each group (Group A: PDG7, PDG8, PDG10 and PDG16; Group B: PDG13, PDG17, PDG31 and PTG37 [sic]) were analysed by automated sequencing and compared with known strain line gene sequences (Tables 3 and 4). There was 100% homology in the deduced amino acid sequences of the H and L chains within each group. By contrast, the homology between Group A and Group B was only 36.9% in the case of the H chain and 81.9% in the case of the L chain amino acid sequences.

In the H chain, Group A clones exhibit the highest degree of sequence identity with the strain line gene VH4.11 of the V_H4 family (Sanz, et al. EMBO J. 8 (1989), 3741-3748). There were 7 amino acid differences in the framework region (FR) and 8 in the complement-determining [sic] region (CDR). Group B clones differed from the mostly homologous 1.9III strain line sequence of the V_H3 family (Berman et al., EMBO J. 7 (1988), 727-738) in four amino acids in the FR and one in the CDR.

In the L chain, the Group A and Group B clones exhibited the highest homology with the DPL2 strain line gene sequence of the $V_λ1$ family (Williams and Winter, Eur. J. Immunol. 323 (1993), 1456). There were nine amino acid differences in FR and ten in CDR in the case of the Group A clones, and one in FR and two in CDR in the case of the Group B clones. The results which were obtained are summarized in Tables 3 and 4.

Table 3

A. Heavy chains

Clones	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VH4.11	QVQLQESGPGLVKPSETLSLTCTVSGGSIS	SYYWS	WIRQPPGKGLEWIG	YIYYSGSTNYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR		
PDG7	--K-L----------N-----R---	G-S-R	----S----------	D-S-----K-K---R-	---L----------N----------	VLPFDPWGQGTLVTVSS	
PDG8	----------	-----	----------	----------	----------	VLPFDPWGQGTLVTVSS	
PDG10	----------	-----	----------	----------	----------	VLPFDPWGQGTLVTVSS	
PDG16	----------	-----	----------	----------	----------	VLPFDPWGQGTLVTVSS	
1.9III	QVQLVESGGGVVQPGRSLRLSCAASGITFS	SYGMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
PDG13	--K-L----------	--A--	----------	----------	--A----------R	ALGSWGQGTMVTVSS	
PDG17	----------	-----	----------	----------	----------	ALGSWGQGTMVTVSS	
PDG31	----------	-----	----------	----------	----------K----------	ALGSWGQGTMVTVSS	
PDG37	----------	-----	----------	----------	----------	ALGSWGQGTMVTVSS	
M85255	--Q-V----------	-----	----------	----------	--T----------	DRPIAAWTYDGMDV	WGQGTTVTVSS

B. Light chains

Clones	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
DPL2	VLTQPPSASGTPGQRVTISC	SGSSSNIGSNTVN	WYQQLPGTAPKLLIY	SNNQRPS	GVPDRFSGSKSGTSASLAISGLQSEDEADYYC	AAWDDSLNGWV	
PDG7	-V----------H-----	-------R--P-S	--N-V---------T	GSN----	----------R----G-NG----	-T---G---PV	FGGGTKLTVLSQP
PDG8	----------	----------	----------	-------	----------	----------	FGGGTKLTVLSQP
PDG10	----------	----------	----------	-------	----------	----------	FGGGTKLTVLSQP
PDG16	----------	----------	----------	-------	----------	----------	FGGGTKLTVLSQP
DPL7	VLTQPPSASGTPGQRVTISC	SGSSSNIGSNTVN	WYQQLPGTAPKLLIY	SNNQRPS	GVPDRFSGSKSGTSASLAISGLQSEDEADYYC	AAWDDSLNGWV	
PDG13	-V----------	----------	----------	-------	----------	---------HV	FGGGTKLTVLGQP
PDG17	----------	----------	----------	-------	----------	----------	FGGGTKLTVLGQP
PDG31	----------	----------	----------	-------	----------	----------	FGGGTKLTVLGQP
PDG37	----------	----------	----------	-------	----------	----------	FGGGTKLTVLGQP

FR: framework region; CDR: complement-determining [sic] region. The top sequences (VH4.11; 1.9III; DPL2) are given for comparative purposes and in each case represent the deduced amino acid sequence for the most closely related published strainline gene sequence. Dashes denote identity. M85255 refers to the EMPL/GenBank reference number and denotes the deduced amino acid sequence of the human anti-GPIIb autoantibody 2E7 (Kunicki et al., J. Autoimmun. 4 (1991), 433-446). In the case of the heavy chain, the first three amino acids (QVK) are specified by the pComb3 vector sequence.

Table 4 shows the assignment of the Group A and Group B clones to known strainline V gene sequences in accordance with the amino acid homology

	Heavy chain			Light chain		
PDG phab clones	**V_H family**	**Strain-line gene**	**Homology (%)**	**V_λ family**	**Strain-line gene**	**Homology (5)**
Group A: 7,8,10, 16	V_H4	$V_{H4}.11$	84.3	$V_\lambda I$	DPL2	81.4
Group B: 13,17, 31,37	V_H3	1.9III	95.1	$V_\lambda I$	DPL2	97.1

2. Identifying antiidiotypic antibody sequences

2.1 Phab clones AI-X

The phagemids technique was used to identify sequences for antiidiotypic antibodies in accordance with the method described in Example 1. The clone PDG16, which was selected in Example 1, was used as the antigen. There was no negative preselection.

Use was made of a pool of combinatorial phab libraries [lacuna] the specificities of a nonimmune library of peripheral B lymphocytes and of a library of peripheral lymphocytes which had been immobilized with red blood cells, and also of a nonimmune library of B lymphocytes obtained from tonsils.

The pool of phabs which was obtained after the fourth round of panning was analysed. For this, 40 phab clones were selected at random and their binding specificities were determined. 25 of the selected clones reacted with anti-GPIIb/IIIa phab. These antiidiotypic phab clones belong to two groups: Group I (three clones) only

reacted with Group A autoantibody phab clones (PDG 7, 8, 10 and 16), whereas the Group II phab clones (22 clones in all) reacted with the Group A and Group B phab clones, with murine monoclonal anti-GPIIb/IIIa antibodies, with purified serum immunoglobulin (IVIgG) or $F(ab')_2$ fragments thereof, and with anti-IgE Fab. 14 phab clones (Group III) did not react with any of the substances mentioned. One Group IV phab clone only reacted with anti-GPIIb/IIIa antibodies. The results of these specificity assays are summarized in Table 5a.

A DNA sequence analysis carried out on Group I phab clones (AI-X16, 17 and 24) showed complete identity in the heavy-chain-encoding sequences apart from one amino acid in the CDR2 region and complete identity in the light-chain-encoding sequences. A comparison with known strainline gene sequences showed approx. 85% homology with the VH3 H chain sequence and approx. 90% homology with the V-λII L chain family sequence. A DNA sequence analysis of the H chain gene was carried out on one representative of each of the Group II, III and IV phab clones. The results of this sequence analysis, and of the comparison with known strainline gene sequences, are summarized in Tables 6 and 7a.

The result of an inhibition assay is depicted in Fig. 1. The inhibition of the binding of AI-X17 to PDG-A by purified GPIIb/IIIa was determined by means of an immunodot assay. 660 and 220 ng of PDG-A phab, respectively, were added to nitrocellulose. The antigen was incubated for 2 h with GPIIb/IIIa at concentrations in the range from 50 µg/ml to 50 ng/ml, and with a buffer solution as control, and then incubated for a further two hours with the phage clone AI-X17 (final concentration 10^{12}/ml). The bound phages were detected using peroxidase-conjungated polyclonal rabbit anti-phage antibody and electrochemiluminescence.

It was found that the AI-X17 phab (Group I) is able to inhibit the binding of Group A antibody phabs (PDG-X) to the IIb/IIIa glycoprotein. This signifies that AI-X17 recognizes the antigen-binding site on PDG-A.

Another clone AI-X2 which binds to PDG-A was sequenced. Like clones AI-X20, 39 and 40, this clone only has a heavy chain and no light chain. The heavy chain is able to bind on its own, possibly as a dimer, to the antigen, i.e. PDG-A, with adequate specificity and affinity.

2.2 Phab clones AI-B

The phagemid technique was used to identify sequences of other antiidiotypic antibodies in accordance with the method described in Example 2.1. A clone PDG-B which was selected in Example 1 was used as the antigen.

In all, 40 phab clones were selected and their binding specificity determined. 34 of the selected clones reacted with anti-GPIIb/IIIa PHAB. These antiidiotypic phab clones belonged to three groups:

Group I (14 clones) only reacted with the Group B antibody phab clones, whereas the Group II phab clones (8 clones in all) reacted with both Group A and Group B phab clones. The Group III phab clones (12 clones in all) additionally reacted with murine monoclonal anti-GPIIb/IIIa antibodies, with purified serum immunoglobulin (IVIgG) or $F(ab')_2$ fragments thereof, and with anti-IgE Fab. Six phab clones (Group IV) did not react with any of the substances mentioned. The results of these specificity assays are summarized in Table 5b.

The result of carrying out a DNA sequence analysis on Group I phab clones (AI-14, 18, 24 and 38) is summarized in Tables 6 and 7b. Clones AI-B14, 18 and 38 only had a heavy chain.

AI-B14 and 17 are identical. AI-B34 and 40 are likewise identical with AI-B18.

The inhibition of the binding of PDG-B to platelets by AI-B phabs is depicted in Fig. 2. This was determined by means of flow-cytometric analysis. For this, a platelet-rich plasma (10^7 platelets in all) was incubated with biotinylated PDG-B in the presence or absence of AI-B phabs and using helper phages as the control. The platelets were fixed with paraformaldehyde and bound PDG-B was detected with R-phycoerythrin (RPE)-labelled streptavidin. 10,000 events were counted in a FACScan appliance and the mean value of the fluorescence (± SD) was recorded. The strongest inhibition (> 60%) was achieved with clones AI-B18, 24 and 38. The inhibition of the binding shows that AI-B clones interact with the antigen-binding site on PDG-B.

Table 5a

AIX phab clones		Binding to PDG A	PDGB	anti-IgE Fab	anti-GPIIb/IIIa mAb	SG	$F(ab')_2$
Group I							
16,17,24	3	+	-	-	-	-	-
Group II							
1,2,3,4,5,6,7,9,11, 13,14,23,26,27,28,29, 33,35,36,37,38,40	22	+	+	+	+	+	+
Group III							
8,10,12,15,18,19,21, 22,25,30,31,32,34,39	14	-	-	-	-	-	-
Group IV							
20	1	-	-	-	+	-	-

Table 5b

AI-B phab clones

n	Binding to PDG-X	PDG-B	anti-IgE Fab	anti-GPIIb/IIIa mAb	IvIgG	IvIgG F(ab')$_2$
14 (AI-B5,7,8,14,17, 18,23,24,30,31,33, 34,38,40)	-	+	-	-	-	-
8	+	+	-	-	-	-
12	+	+	+	+	+	+
6	-	-	-	-	-	-

Table 6

anti-Id phage clones antiidiotypic phab clones (AI-X and AI-B)	H chain			L chain		
	V_H family	Strainline gene	Homology (%)*	V_λ family	Strainline gene	Homology (%)*
AI-X16, AI-X24	V_H3	DP47	88	$V_\lambda2$	DPL10	88
AI-X17	V_H3	DP47	87	$V_\lambda2$	DPL10	88
AI-X39	V_H3	DP49	94	-	-	-
AI-X40	V_H3	DP31	95	-	-	-
AI-X20	V_H4	DP71	78	-	-	-
AI-B14, AI-B17	V_H3	DP46	91	-	-	-
AI-B18	V_H1	DP10	85	-	-	-
AI-B24	V_H3	DP49	81	$V_\lambda3$	3h	82
AI-B38	V_H1	DP5	98	-	-	-

* Highest homology (in %) of the amino acid sequences of the respective phab clones with sequences of known strainline V genes

Table 7a

A. Heavy chains

Clones	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
DP47	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYAMS	WVRQAPGKGLEWVS	AISGSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
AIX16	Q-K---------H----------D	NF---	----------	G---G-LL-H-------	------N--R--V----------	VRDLGYRVLSTFTFDI	WGQGTKVTVSS
AIX24	----------	-----	----------	----------	----------	----------	----------
AIX17	----------	-----	----------	----------N----	----------	----------	----------
DP49	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
AIX39	--K-L-------H----------	--T--	----------	L----------	--A----------K----------	DGRSGSYARFDGMDV	WGQGTTVTVSS
DP31	EVQLVESGGGLVQPGRSLRLSCAASGFTFD	DYAMH	WVRQAPGKGLEWVS	GISWNSGSIGYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTALYYCAKD		
AIX40	Q-K-L----------	---L-	----------	----D-T----------	----------V--	HGSSVVATYNAFDI	WGQGTHVTVSS
DP71	QVQLQESGPGLVKPSETLSLTCTVSGGSIS	SYYWS	WIRQPPGKGLEWIG	YIYYSGSTNYNPSLKS	RVTISVDTSKNQFSLKLSSVTAADTAVYYCAR		
AIX20	--K-L----------DV--R	-N---	-L----------	F--DGAR-RF----R-	--SL-M-P-K-------G------S-------	DADGDGFSPYYFDY	WGQGIPVSVSS

B. Light chains

Clones	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
DPL10	QSALTQPASVSGSPGQSITISC	TGTSSDVGSYNLVS	WYQQHPGKAPKLMIY	EVSKRPS	GVSNRFSGSKSGNTASLTISGLQAEDEADYYC	CSYAGSSTF	
AIX16	VV----------	-----AI-N--F-P	----------	-G-----	----------E---	---VH---N	HVFGGGTKLTVLGQPKAAPSVTLFPPSS
AIX24	----------	----------	----------	-------	----------	----------	----------
AIX17	----------	----------	----------	-------	----------	----------	----------
25							

FR: framework region; CDR: complement-determining [sic] region. The top sequences (DP47, DP49, DP31, DP71 and DPL10) are given for comparative purposes and represent the most closely related known strainline sequences. Dashes denote identity. In the case of the heavy chain, the first three amino acids (QVK) are specified by the pComb3 vector sequence.

Table 7b

A. Heavy chains

Clones	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
DP-46	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
AI-B14	--K-L----------	D-G--	----------	A----------	--S------N--------ST---------F---	DSETAIFAAAGRFDI	WGQGTMVTVSS
AI-B11	----------	-----	----------	----------	----------	----------	----------
DP-10	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS	SYAIS	WVRQAPGQGLEWMG	GIIPIFGTANYAQKFQG	RVTITADESTSTAYMELSSLRSEDTAVYYCAR		
AI-B18	--K-LE----------H----------	-MT--	----------	--T-----V--------	--------P--------R--T-DDSGI-----	EDGTTVPSQPLEF	WGQGTRVTVSS
DP-49	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
AI-B24	--K-L-----L----G------S------N	K-AI-	----------Y-S	A--SN-G-T--------	----------V----S----------VR	GSGSYLGYYFDY	WGQGTLVTVSS
DP-5	QVQLVQSGAEVKKPGASVKVSCKVSGYTLT	ELSMH	WVRQAPGKGLEWMG	GFDPEDGETIYAQKFQG	RVTMTEDTSTDTAYMELSSLRSEDTAVYYCAT		
AI-B38	Q-K-LE----------	-----	----------	----------	----------	GLRSYNYGRHLDY	WGQGTLVTVSS

B. Light chains

Clones	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VL3h	SYVLTQPPSVSVAPGKTARITC	GGHNIGSKSVH	WYQQKPGQAPVLVIY	YDSDRPS	EIPERFSGSNSGNTATLTISRVEAGDEADYYC	QVWDSSSDH	
AI-B24	-V----------RQ--T---	--YK------	----------V-	E--Y---	E----------N-----TG----------	----NTH-Q	TIFGGGTKLTVLRQPKAAPSVTLFPPSS

FR: framework region; CDR: complement-determining [sic] region. The top sequences (DP46, DP10, DP49, DP5 and VL3h) are given for comparative purposes and represent the most closely related known strainline sequences. Dashes denote identity. In the case of the heavy chain, the first three amino acids (QVK) are specified by the pComb3 vector sequence.

3. Effect of autoantibody polypeptides on the binding of fibrinogen to blood platelets

3.1 Methods

Analysis of the binding of Fab to EDTA-pretreated blood platelets

A blood platelet-rich plasma was incubated with 10 mM EDTA for 30 min. Biotinylated PDG-B and PDG-A polypeptides were added and the mixture was incubated at room temperature for 1 h. The binding of PDG-A and PDG-B to blood platelets was measured by flow-cytometric analysis using phycoerythrin-labelled streptavidin.

Aggregation experiments

Blood platelet-rich plasma (250 x 10^9/l) was prepared freshly and maintained under 5% CO_2. The plasma was activated by different dilutions of ADP (maximum concentration 410 µM) in the absence or presence of PDG-A or PDG-B (maximum quantity 10 µg of Fab). The aggregation was measured in a Rodell 300BD-5 aggregometer (Baxter AG, Düdingen, Switzerland). In subsequent experiments, polyclonal anti-Fab antiserum was added to the activated platelets after PDG-A or PDG-B had been added.

Fibrinogen binding test

Wells of ELISA plates were coated with 0.5 µg/ml GPIIb/IIIa and blocked with 3.5% bovine serum albumin in Tris-buffered salt solution. Fibrinogen (Kabi Diagnostics, Stockholm, Sweden) was then added at different concentrations (maximally 0.08 µg/ml) in the absence or in the presence of PDG-A, PDG-B or anti-IgE Fab as the control (maximum concentration 23 µg/ml).

The bound fibrinogen was measured with rat anti-human fibrinogen antibody, biotinylated mouse anti-rat antibody and a conjugate consisting of streptavidin and biotinylated horseradish peroxidase (Amersham Pharmacia Biotech Europe GmbH, Dübendorf, Switzerland) and using an ELISA Easy Reader (EAR340AT, SLT Instruments, Austria) at 405 nm.

Competition assay using the monoclonal antibody 7E3 and the antibody fragment ReoPro®

Platelet-rich plasma (230 × 10^9/l) was incubated for 1.5 h with PDG-B or PDG-A (200 and 400 µg/ml, respectively) with or without the murine monoclonal antibody 7E3 or its Fab fragment ReoPro® (total quantity of Fab in the range from 10^{14} to 10^{10}). After fixing with an equal volume of 1% paraformaldehyde, the binding of PDG-B and PDG-A to platelets was measured by flow-cytometric analysis using phycoerythrin-labelled streptavidin.

3.2 Results

The recombinant anti-GPIIb/IIIa Fab autoantibody fragments which were tested do not exhibit any binding to blood platelets which had been pretreated with 10 mM EDTA. This shows that the autoantibody fragments only recognize an antigen whose confirmation is intact (Fig. 3).

In aggregation experiments using platelet-enriched plasma, neither PDG-A nor PDG-B inhibited the aggregation. In a fibrinogen-binding test in which the concentration of fibrinogen was from 10^4 to 10^6 times lower than in serum, PDG-A and PDG-B completely inhibited the fibrinogen binding (Fig. 4). No inhibition occurred when anti-IgE Fab, which had been obtained by a similar enrichment protocol, was used as

the control. These results show that both PDG-A and PDG-B interact powerfully with the fibrinogen-binding site on GPIIb/IIIa.

In investigations carried out with the murine monoclonal anti-GPIIb/IIIa antibody 7E3 and its commercially available Fab fragment ReoPro®, both of which inhibit the binding of fibrinogen to activated GPIIb/IIIa, the binding of PDG-B to blood platelets was found to be inhibited selectively and completely (Figures 5 to 7). By contrast, the binding of PDG-A to blood platelets was not inhibited significantly either by 7E3 or by ReoPro®.